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Molecules and Cells

ADAM7 Is Associated with Epididymosomes and Integrated into Sperm Plasma Membrane

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During epididymal transit, mammalian sperm acquire selected proteins secreted by the epididymis. We previously showed that a disintegrin and metalloprotease (ADAM) 7 is expressed specifically in the epididymis and transferred to the sperm surface during epididymal transit. Here, we show that mouse ADAM7 secreted to the epididymal lumen is associated with membranous vesicles known as epididymosomes. Furthermore, we found that ADAM7 can be transferred directly from epididymal vesicles to sperm and that it is an integral plasma membrane protein in sperm. Thus, our study provides new information regarding the unique mode of secretion and interaction of ADAM7 during the epididymis-to-sperm transfer process.

INTRODUCTION

Mammalian sperm acquire their forward motility and the ability to fertilize an egg during their transit through the epididymis, which is a highly convoluted tubule that conveys sperm from the testis to the vas deferens. These maturational events are made possible by the particular luminal microenvironment created by the secretory activities of the bordering epithelial cells of the epididymis. The principal components of this environment are specific proteins synthesized and secreted in certain regions of the epididymis. Sperm maturation occurring in the epididymis depends on interaction of these proteins with the sperm surface. Some of these proteins are secreted and transferred to the sperm surface, where they behave like integral membrane proteins (Cooper, 1998), which suggests the presence of an unusual mechanism underlying epididymal protein secretion and protein interaction at the sperm surface (Kirchhoff and Hale, 1996).

Besides the classical secretory exocrine process, several studies have found that the epithelial cells lining the epididymis are involved in an apocrine secretion mechanism (Aumuller et al., 1997; 1999). Apocrine secretion results in the formation of cell blebs that will detach into the intraluminal compartment. The apical blebs disintegrate, liberating their content, including small membranous vesicles known as epididymosomes (Aumuller et al., 1997; 1999; Hermo and Jacks, 2002). These vesicles have been described in several species, including humans (Frenette et al., 2005), horses (Arienti et al., 1998), sheep (Gatti et al., 2005), chimpanzees (Frohlich and Young, 1996), hamsters (Legare et al., 1999), bulls (Frenette and Sullivan, 2001; Frenette et al., 2002; 2003) and mice (Rejraji et al., 2002; 2006). *In vitro* and *in vivo* studies have shown that these vesicles, which are present in the cauda epididymis and seminal plasma, transfer a number of proteins to sperm (Frenette and Sullivan, 2001; Frenette et al., 2002; 2003; Rejraji et al., 2002). Additionally, some of these proteins have been shown to be essential for sperm motility and fertility (Frenette et al., 2005; 2006; Legare et al., 1999; Sullivan et al., 2005).

A disintegrin and metalloproteases (ADAMs) are a family of transmembrane proteins with a unique domain structure composed of a signal sequence, pro-, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic tail domains (Edwards et al., 2008; Jin et al., 2007; Primakoff and Myles, 2000; Seals and Courtneidge, 2003). The ADAM family currently has at least 33 members, more than half of which are known to be expressed in mammalian male reproductive organs (Cho, 2005). Among these reproductive ADAMs, only ADAM7 is expressed specifically in the epididymis (Cornwall and Hsia, 1997; Oh et al., 2005; Perry et al., 1992). The intriguing feature of ADAM7 is its relocation to sperm and its relationship with other sperm surface ADAMs. Previously, we and others have shown that ADAM7 protein synthesized in the epididymis is transferred to the sperm surface in mice and humans (Lin et al., 2001; Liu et al., 2000; Oh et al., 2005; Sun et al., 2000). We also recently found that more than half of ADAM7 is lost in sperm from mice containing a deletion of the gene encoding ADAM2 or ADAM3, which implicates ADAM7 in ADAM2- and ADAM3-dependent association with sperm (Kim et al., 2006). The ADAM2 and ADAM3 proteins synthesized in testicular spermatogenic cells are present at the surface of epididymal sperm and play critical roles in the fertilization process (Cho et al., 1998; Nishimura et al., 2001; 2004). Thus, the addition of epididymal ADAM7 to sperm might be related to acquisition of the fertilizing ability of sperm.

The present study was conducted to investigate how mouse ADAM7 is secreted to the epididymal lumen and bound to the sperm surface. We found for the first time that ADAM7 is associated with membranous vesicles in the epididymal lumen rather than being secreted in a soluble form. We further provide

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new information about the nature of the interaction of ADAM7 with the sperm plasma membrane.

MATERIALS AND METHODS

Antibodies

Affinity-purified polyclonal antibodies against the cytoplasmic tail domain of ADAM7 and ADAM28 were prepared as previously described (Oh et al., 2005). Monoclonal anti-mouse ADAM2 (9D2.2) and polyclonal anti-caveolin-1 antibodies were purchased from Chemicon and Santa Cruz Biotechnology, respectively.

Preparation of samples

Eight to twelve week-old male ICR mice were sacrificed. All animal investigations were carried out according to the guidelines of the Animal Care and Use of Gwangju Institute of Science and Technology. Epididymal sperm were isolated from the caput, corpus or cauda epididymis. Epididymal fluid was obtained by retrograde flushing by applying air pressure with a syringe inserted into the vas deferens. These procedures were applied with great care to avoid blood or tissue contamination. The epididymal fluid was then diluted with 150 mM NaCl, centrifuged twice at 1,000 × *g* for 10 min to remove the sperm and once at 12,000 × *g* to remove residual sperm and the remaining debris. The resulting supernatant was observed under a microscope to confirm the absence of residual sperm and epididymal tissues, after which it was ultracentrifuged at 100,000 × *g* for 2 h. After centrifugation, the supernatants were pooled and collected as an epididymal fluid excluding epididymal vesicles. Additionally, the pellet was suspended in 150 mM NaCl and centrifuged again at 100,000 × *g* for 2 h. The resulting pellets, which were considered to be the epididymal vesicles or epididymosomes, were resuspended in PBS and stored at -20°C until analysis (Frenette et al., 2002). Each sample was constituted with five animal epididymides. Biotinylation and trypsinization were then performed as previously described (Oh et al., 2005).

Immunoblot analysis

Proteins were denatured by boiling for 5 min in 1X SDS sample buffer containing 5% β-mercaptoethanol, after which they were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were then blocked in TBS-T containing 5% nonfat dried milk, after which they were incubated with primary antibodies and rinsed. The bound IgG was then detected with alkaline phosphatase-conjugated secondary antibodies (Jackson Immunoresearch) and the alkaline phosphatase activity was detected by NBT/BCIP (Promega Biotech).

Glycosidase digestion

Lysates from epididymal cells, sperm and epididymal vesicles were denatured in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) at 100°C for 10 min, after which they were incubated in G5 buffer (50 mM Sodium Citrate, pH 5.5) with Endoglycosidase Endo H (New England Biolabs) at a concentration of 50,000 units/ml or in G7 buffer (50 mM Sodium Phosphate, 1% NP-40, pH 7.5) with Endoglycosidase PNGase F (New England Biolabs) at a concentration of 50,000 units/ml at 37°C for 3 h.

Incubation of sperm with biotinylated epididymosomes

Epididymosomes were biotinylated and incubated with caput or testicular sperm in a solution consisting of 150 mM NaCl and 1 mM $ZnCl_2$ (pH 6.5) at 37°C for 3 h with gentle agitation. At the end of the incubation period, sperm were centrifuged at 1,000 × *g* for 10 min and then washed twice with isotonic NaCl. Caput sperm were freshly prepared and incubated in 150 mM NaCl or M2 medium at 37°C for 3 h with gentle agitation in the absence or presence of 1 mM $ZnCl_2$ at pH 6.5. After incubation, cell death was assayed using trypan blue staining.

ADAM7 extraction from sperm

Sperm from the epididymis were suspended in PBS containing protease inhibitors at room temperature and then centrifuged at 1,000 × *g* for 10 min. The sperm suspension was then divided into four equal parts. Next, each part was resuspended in 150 mM NaCl, 500 mM NaCl, 0.1% Triton X-100 or 1% SDS for 30 min at room temperature. The samples were then centrifuged at 1,000 × *g* for 10 min, after which the supernatants were centrifuged twice at 12,000 × *g* at 4°C for 10 min to remove the cell debris and residual sperm, and the pellets were washed twice with PBS. The supernatants and pellets were then subjected to immunoblot analysis.

Phsophatidylinositol-specific phospholipase C (PI-PLC) treatment of sperm

Sperm were suspended in buffer consisting of 10 mM Tris-Cl, pH 7.4, 144 mM NaCl and 0.05% BSA with or without 1U of PI-PLC (Sigma) at 30°C for 30 min. The sample was then centrifuged at 1,000 × *g* for 10 min, after which the supernatant was further centrifuged at 12,000 × *g* at 4°C for 10 min to eliminate cell debris and residual sperm. Next, the pellets were washed twice with PBS, after which the supernatants and pellets were subjected to immunoblot analysis.

Sperm immunofluorescence

Cauda epididymal sperm were fixed on glass slides in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 or left untreated. After extensive washing with PBS, the sperm were incubated with normal goat serum at room temperature for 30 min and then exposed to the ADAM7 antibody (1:100 in PBS) overnight at 4°C. Next, the sperm were washed three times in PBS and incubated at room temperature with Rhodamine Red-X goat anti-rabbit IgG (1:1000 in PBS; Molecular Probes) for 30 min. After washing three times in PBS, sperm fluorescence was observed under a microscope (DMLB; Leica Microsystems).

RESULTS

To determine how ADAM7 synthesized in the epididymis is secreted and transferred to the sperm surface, we performed Western blot analysis on various samples prepared from the mouse epididymis, including epididymal somatic cells, total epididymal fluid, epididymal sperm, membranous vesicles (also known as epididymosomes) present in intraluminal fluid from the epididymis and epididymal fluid excluding the vesicles. Consistent with a previously conducted study (Oh et al., 2005), ADAM7 was present in the epididymal cells and sperm in the present study. Additionally, the results of this study demonstrate for the first time that ADAM7 is present in epididymal vesicles, but not in epididymal fluid without the vesicles (Fig. 1A). However, ADAM28 and ADAM2, which are an epididymal protein and sperm membrane protein, respectively (Kim et al., 2006; Oh et al., 2005), were not present in the epididymal fluid or vesicles. Additionally, immunoglobulin G, which is a luminal protein in the epididymis (Weininger et al., 1982), was present only in the epididymal fluid. Caveolin-1, previously found in prostasomes, membranous vesicles similar to epididymosomes (Llorente et al., 2004), was detected in the epididymal vesicles.

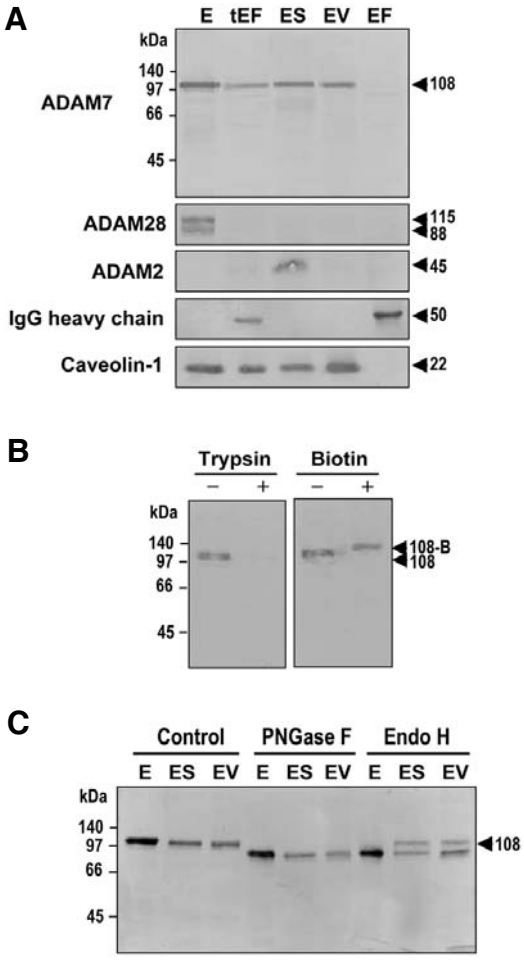

Fig. 1. Association of ADAM7 with epididymal vesicles. (A) Various epididymal samples were boiled in 3% SDS with 5% β-mercaptoethanol and then analyzed by Western blot using the anti-ADAM7 cytoplasmic domain antibody, anti-ADAM28 cytoplasmic domain antibody, anti-ADAM2 antibody, anti-caveolin-1 antibody and anti-mouse IgG antibody. E, epididymal somtic cells; tEF, total epididymal fluid; ES, epididymal sperm; EV, epididymal vesicles or epididymosomes; EF, epididymal fluid excluding epididymal vesicles. (B) Epididymal vesicles were (+) or were not (-) treated with biotin or trypsin. Epididymal vesicles were retrieved, subjected to SDS-PAGE and then analyzed by Western blot using anti-ADAM7 antibody. (C) Protein samples were treated with the endoglycosidases Endo H or PNGase F, subjected to SDS-PAGE and then analyzed by Western blot with anti-ADAM7 antibody. E, epididymal somatic cells; ES, epididymal sperm; EV, epididymal vesicles.

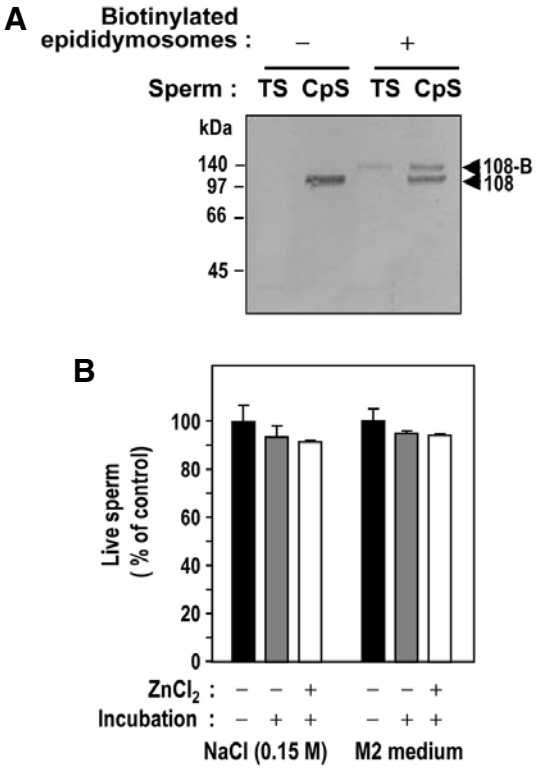

Fig. 2. *In vitro* transfer of ADAM7 from epididymal vesicles to sperm. (A) Testicular or caput sperm were (+) or were not (-) incubated with biotinylated epididymal vesicles. Sperm were retrieved and subjected to SDS-PAGE and then analyzed by Western blot with the anti-ADAM7 antibody. TS, testicular sperm; CpS, caput sperm. (B) Caput sperm were incubated with (+) or without (-) 1 mM $ZnCl_2$ for 3 hr in 150 mM NaCl or M2 medium. Live sperm were counted using trypan blue staining prior to (-) or after (+) incubation. Each bar shows the mean percentage of the control ± SD based on measurements from three independent experiments.

Thus, these results indicate that the vesicles from the epididymal fluid were free from epididymal tissues and sperm. To analyze the association of ADAM7 with epididymal vesicles, we performed surface labeling and trypsinization experiments. Surface labeling of epididymal vesicles with biotin resulted in an increase in the molecular size of ADAM7 and the protein was affected by surface trypsinization of the epididymal vesicles, suggesting the presence of ADAM7 at the surface of the epididymal vesicles (Fig. 1B).

To gain further information regarding the secretion pattern of ADAM7, we performed glycosidase digestion of the protein in epididymal cells, vesicles and sperm. Endo H was used to distinguish the complex oligosaccharides from high-mannose oligosaccharides because it processes only high-mannose carbohydrates in glycoproteins that have not passed through the medial-Golgi apparatus. In contrast, PNGase F digests all types of N-linked carbohydrates. We found that the carbohydrate moieties of ADAM7 are sensitive to Endo H in epididymal cells and partially resistant to the enzyme at the same concentration as is present in epididymal vesicles and sperm (Fig. 1C). The absence and presence of Endo-H-sensitive ADAM7 prior to and after secretion, respectively, implies an unusual mode of secretion of the protein in the epididymis (see "Discussion").

We next determined if ADAM7 associated with the epididymal vesicles is directly transferred to sperm. Previously, it was demonstrated that co-incubation of caput sperm with cauda epididymal vesicles under a physiologically-relevant *in vitro* condition resulted in the transfer of several selected proteins from the epididymal vesicles to the sperm (Frenette et al., 2002). We labeled the surface of epididymal vesicles with biotin and co-incubated them with testicular or caput sperm. Western blot analysis revealed the presence of biotinylated ADAM7 protein in both the testicular and caput sperm (Fig. 2A). Furthermore, the acquisition of ADAM7 from the epididymal vesicles was found to be more efficient in caput sperm than testicular sperm (Fig. 2A). It should be noted that cell death did not

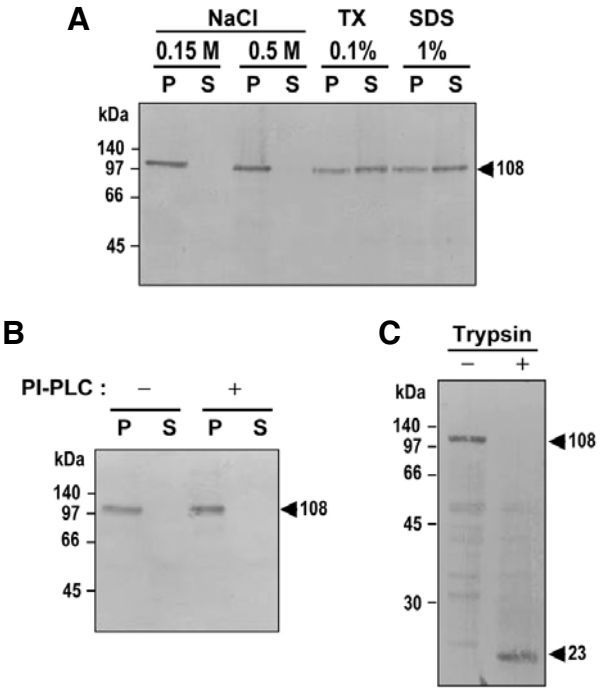

Fig. 3. Interaction of ADAM7 and sperm. (A) Epididymal sperm were treated with NaCl (0.15 or 0.5 M), Triton X (TX)-100 (0.1%) or SDS (1%) and then centrifuged to separate the pellet and supernatant. Each fraction was boiled in 3% SDS with 5% β-mercaptoethanol, subjected to SDS-PAGE and then analyzed by Western blot with anti-ADAM7 antibody. P, pellet after centrifugation; S, supernatant after centrifugation. (B) Epididymal sperm were (+) or were not (-) treated with PI-PLC and then centrifuged to separate the pellet and supernatant. Each fraction was boiled in 3% SDS with 5% β-mercaptoethanol, subjected to SDS-PAGE and then analyzed by Western blot with the anti-ADAM7 antibody. P, pellet after centrifugation; S, supernatant after centrifugation. PI-PLC, phosphatidylinositol-specific phospholipase C. (C) Epididymal sperm were (+) or were not (-) treated with trypsin. Lysates from sperm were boiled in 3% SDS with 5% β-mercaptoethanol, subjected to SDS-PAGE and then analyzed by Western blot with the anti-ADAM7 antibody.

occur during incubation in the presence or absence of zinc ions, indicating that the condition of the incubation was not toxic to the sperm (Fig. 2B). Taken together, these results strongly suggest that ADAM7 is relocated from epididymal cells to sperm directly through epididymal vesicles.

To gain insight into how ADAM7 on epididymal vesicles interacts with and undergoes relocation to the sperm surface, we analyzed the nature of the interaction of ADAM7 with the sperm surface. When epididymal sperm were treated with isotonic or hypertonic salt solution, ADAM7 was found to be present only in the sperm pellets (Fig. 3A). Furthermore, treatment of sperm with detergents such as Triton X-100 and SDS did not completely release ADAM7 from the sperm (Fig. 3A), revealing a firm association of ADAM7 with sperm. A number of proteins associated with epididymal vesicles that are transferred to sperm are known to be glycosylphosphatidylinositol (GPI)-anchored to the sperm plasma membrane (Kirchhoff and Hale, 1996; Sullivan et al., 2005). To determine if this is the case for ADAM7, epididymal sperm were treated with PI-PLC. As shown in Fig. 3B, this treatment did not induce the release of ADAM7 from the sperm surface, indicating that the association of ADAM7 with sperm does not occur through GPI-anchoring. Previously, it was demonstrated that ADAM7 is transferred from epididymal cells with the intact transmembrane domain to the plasma membrane over the entire sperm head (Oh et al., 2005). In that study, it was suggested that the cytoplasmic domain of ADAM7 was located at the surface of the sperm based on the observation that no protein bands were detected by antibody to the cytoplasmic domain in sperm treated with trypsin (Oh et al., 2005). Nonetheless, we cannot exclude the possibility that ADAM7 is an integral membrane protein, since the trypsin-resistant region (transmembrane and cytoplasmic domains), of which expected molecular mass is about 20 kDa, could be too small to be detected in the immunoblot in the previous study. Thus, we performed immunoblot analysis of trypsinized sperm using a gel with a low molecular weight range. As shown in Fig. 3C, the 23-kDa protein detected by the antibody to the cytoplasmic domain was protected from trypsinization, suggesting that the domain was localized in the intracellular region of the sperm. We also conducted indirect immunofluorescence to corroborate this finding. We observed bright fluorescent staining in the heads of all of the permeabilized epididymal sperm using the antibody to the cytoplasmic domain of ADAM7 (Fig. 4), consistent with our previous finding (Oh et al., 2005). Conversely, no fluorescent signal was detected in sperm with intact plasma membranes using the same antibody (Fig. 4). These results suggest that ADAM7 is embedded in the sperm membrane where the hydrophilic, carboxy-terminal domain protrudes into the environment inside the sperm cell.

DISCUSSION

Previously, we demonstrated that mouse ADAM7 is synthesized in the epididymis and transferred to the sperm surface. The unique feature of ADAM7 in this process is that relocation of the protein to sperm occurs without the removal of the transmembrane domain (Oh et al., 2005). In the present study, we identified the atypical glycosylation pattern of ADAM7 and the association of the protein with membrane vesicles in the epididymal lumen. Our data showed that all of the ADAM7 molecules are susceptible to Endo H in epididymal cells, and that about half of them are resistant to the enzyme in epididymal vesicles and sperm. This unusual dual sensitivity might reflect the existence of major stores of ADAM7 in the endoplasmic reticulum and the rapid release of the protein from the cells into the epididymal lumen following passage of the protein through the Golgi, where Endo H resistance in at least some of the protein may be acquired. With regard to the process of ADAM7 release, our results are consistent with a model in which ADAM7 is released in the form of membrane vesicles after normal incorporation into the plasma membrane of epididymal cells. In this case, once ADAM7 is incorporated into the plasma membrane, the protein can be internalized into vesicles, which are then transformed into multivesicular bodies and fused with the plasma membrane. This fusion leads to release of the internal vesicles into the epididymal lumen as exosomes. Alternatively, epididymal vesicles might be formed by pinching-off of the apical membrane where ADAM7 is incorporated.

It is important to note that apocrine secretion by the epididymal epithelium is related to ADAM7. Apocrine release occurs in sex- or reproduction-related glands, such as the prostate, mammary glands and epididymis (Aumuller et al., 1999; Gesase and Satoh, 2003; Hermo and Jacks, 2002; Kravets et al., 2000; Saez et al., 2003; Sullivan et al., 2005) and is characterized by protrusion of a part of the apical cytoplasm of cells.

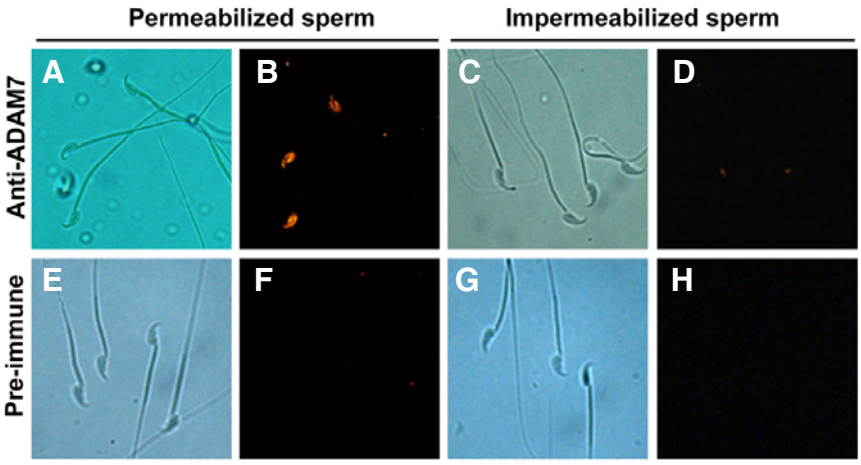

Fig. 4. Sperm immunofluorescence of ADAM7. Epididymal sperm were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (A, B, E, and F) or impermeabilized (C, D, G, and H) and then incubated with the anti-ADAM7 antibody (A-D) or pre-immune serum (E-H), followed by incubation with rhodamine-conjugated goat anti-rabbit IgG. Micrographs show transmission images (A, C, E, and G) and fluorescence images (B, D F, and H). No specific signal was detected in impermeabilized sperm. Magnification 400x.

These protrusions, called apical blebs, contain membranous vesicles of various sizes in the epididymis. The blebs detach from the cell surface and secretory materials are released when they undergo fragmentation. The released materials then either become associated with the vesicles or dissolve in the extracellular region. Thus, apocrine secretion is accompanied with the loss of a portion of the cytoplasm.

Although we do not know the exact molecular mechanism responsible for ADAM7 transfer from epididymal vesicles to sperm, our results imply that the transfer is direct. *In vitro* analyses have shown that protein transfer is pH and temperature dependent, and that zinc at concentrations similar to those found in the epididymis enhances the efficiency of this transfer (Frenette et al., 2002). We found that ADAM7 is transferred to sperm under *in vitro* conditions physiologically similar to the luminal environment of the epididymis. Only a handful of proteins associated with epididymal vesicles have been identified, including HE5, glutathione peroxidase type 5, macrophage migration inhibitory factor, aldose reductase and P26h (Frenette et al., 2003; Kirchhoff and Hale, 1996; Legare et al., 1999; Rejraji et al., 2002; Sullivan et al., 2005). Two of these proteins (HE5 and P26h) are known to be GPI-anchored to the sperm surface during the epididymal transit. It is therefore possible that an identical mechanism is involved in binding of ADAM7 to the sperm membrane. However, sequence analysis of ADAM7 revealed that the ADAM7 carboxy-terminal hydrophobic sequence would be too short to allow for the involvement of the GPI-anchoring mechanism (Udenfriend and Kodukula, 1995). Consistent with the results of the sequence analysis, we found that ADAM7 is not associated with sperm through GPI-anchoring.

Transfer of an integral membrane protein such as ADAM7 from epididymal vesicles to sperm might be mediated by fusion between the vesicle and sperm, or by a process in which epididymal vesicles are removed by endocytosis and the protein is sent back to the sperm plasma membrane (Saez et al., 2003). If so, the structure and composition of the sperm plasma membrane would be important for the acquisition of specific proteins from epididymal vesicles. It has been suggested that lipid raft microdomains are involved in the process of epididymal protein transfer via epididymal vesicles (Sullivan et al., 2005). Lipid rafts are cholesterol- and sphingolipid-enriched microdomains that are present in the plasma membrane of almost all mammalian cells (Brown and London, 2000; Edidin, 2003). The cholesterol/phospholipids ratio is relatively high and close to 2 in epididymosomes; therefore, it is reasonable to postulate that these vesicles are involved in sperm plasma membrane cholesterol transport and this cholesterol is a major source of lipid rafts on the surface of the sperm plasma membrane. Thus, ADAM7 might be involved in lipid rafts in epididymosomes and transfer to specific microdomains of the sperm plasma membrane. In this regard, it should be noted that ADAM7 present on the entire sperm head is redistributed to the posterior region of the sperm head during acrosome reaction. Furthermore, we recently found that the level of ADAM7 is reduced in *Adam2* and *Adam3* knockout sperm, indicating that ADAM2- and ADAM3-depedent association of ADAM7 with sperm occurs during epididymal transit (Kim et al., 2006). Indeed, a portion of ADAM3 was previously found to be present in microdomains (Nishimura et al., 2001).

In conclusion, changes in sperm to acquire motility and fertilizing capacity are related to the acquisition of particular proteins from the epididymis. ADAM7 is an epididymal protein that is transferred to sperm. The level of ADAM7 that is found on sperm is dependent on the expression of ADAM2 and ADAM3, which play critical roles in fertilization. Our analysis of ADAM7 in the mouse epididymis revealed that the transfer of the protein from the epididymis to sperm involves epididymal vesicles. Further studies should be conducted to elucidate the detailed molecular mechanism underlying the interaction between ADAM7 on epididymal vesicles and sperm.

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